I thought it might be helpful to post an expert's review of the concerns raised in the Labaton Sucharow Citizen Petition (CP) regarding western blot (WB) data for Cassava Science's Alzheimer's drug Simufilam.

Simufilam has demonstrated remarkable improvement in cognition in Alzheimer's patients at 12 months of treatment in an open label study and the drug is entering two pivotal phase III studies. By way of introduction, I received a Ph.D. degree in Molecular Biology and have been an academic researcher since 2003. My laboratory studies cancer and other human diseases and we routinely run western blots (~1,000/year) for our studies.

Western blotting is a research tool that we use to analyze proteins, including their levels, modifications, and association with other proteins. Protein extracts are prepared from cells or tissue, denatured using a detergent and heating, and then loaded onto a gel, which is a matrix of polyacrylamide, and then a current applied. This allows for proteins to be separated based on their size (i.e. molecular weight (MW)). After the gel is "run", the proteins are transferred (i.e. blotted) onto a membrane and then probed with an antibody specific for whatever protein you want to analyze. The blot is then probed again, this time with a secondary antibody that is enzyme-linked and binds to the first antibody, thus allowing for detection using a chemiluminescent reagent that produces light where the first antibody binds the membrane (thus producing what is known as a "band"). All WBs include analysis of a control protein (e.g. actin, GAPDH) to confirm that equal amounts of protein extract were loaded in each lane.

The first thing to understand about WBs is that they are semi-quantitative. They can basically tell you if there are differences in levels (increases, decreases) or presence of a modification or binding protein. Secondly, the western blotting technique is somewhat of an artform. It takes practice knowing what procedures are best to analyze a specific protein. Thirdly, there are a lot of variables. Researchers use different first and second antibodies, gel conditions, membrane supports, washing and exposing conditions. There is no standardized protocol. For example, many academic labs that are on a budget will pour their own gels as opposed to buying more expensive pre-made gels. With the former, you have more control over your analysis, but the WBs can often look a little dirtier, lanes slightly unevenly spaced, or bands looking not so sharp. Though the results, in terms of levels etc., should be the same no matter what variations are used.

The majority of concerns raised are easily explained and a small number of others are obvious errors that likely occurred during generating figures for publication. There is no obvious evidence of systematic data manipulation or scientific misconduct. Someone with knowledge of molecular biology likely helped put together the CP, but many of the concerns raised show a shallow understanding of WB technique and data analysis. Below, I provide responses to general concerns and then a point-by-point analysis of each concern raised in the CP.

General concerns raised in the CP:

1. It is stated that "the western blot data presented by Wang/Burns are almost always overexposed and highly processed, which has been repeatedly seen in previously reported examples of image manipulation." Several of the studies scrutinized in the CP were published between 2005-2012. It is important to realize that during this time WBs were visualized by exposing the membrane to film. Control proteins generally give very strong signals due to their high level of expression in cells and use of validated antibodies that give the best (i.e. strongest) signal. Also, analysis of low-expressed proteins requires more extract to be run per lane to see the signal. These factors can often lead to control bands that are strong (i.e. overexposed). Today, most researchers use digital imaging systems to expose WBs, which avoids issues with overexposure. As far as the claim of "highly processed" images, know that most research papers published pre-2010 used images of 300-600 dpi or lower. Images retrieved from online papers, as was done for the CP, are likely even lower resolution. In my opinion, it is dangerous to take a low-resolution WB and adjust the contrast and brightness levels to attempt to uncover evidence of data manipulation. For example, the CP repeatedly shows changes in background pixels as evidence of data manipulation, but there are many factors that could give this effect. Over the years, I've seen things as simple as streaks on a film caused by the processor or how the membrane is wrapped with layers of plastic wrap to prevent drying from influencing the background of the figure.

2. It is repeatedly said in the CP that the authors should produce the original WB data in order to satisfy their concerns. I'm pretty sure the scientists behind the CP know that this request is unreasonable. Most researchers will store data after publication in case questions do arise. In a perfect world, films and electronic WB data would be stored forever, but students leave the lab, labs downsize or move to new institutes, and thumb drives get lost. In addition, journals generally don't require researchers to keep data for longer than a year or so. The fact that Drs. Wang/Burns have not "answered" the WB concerns of the CP is likely not due to them hiding something but rather that it is impossible to retrieve data used in a publication 10-15 years ago. Journals rely on a thorough analysis of the data by the reviewers, which are generally 3 experts in the field. If a reviewer had a problem with any of the WBs in question, they could have asked for them to be repeated. If they thought the data were manipulated in any way, the reviewers would have instantly rejected the paper and likely notified the journal.

3. The CP repeatedly says that their analysis indicates fraud or scientific misconduct. Scientific misconduct is a very serious claim. As scientists, we welcome critiques of our data presented in research papers or presentations. That is part of being a scientist. But know that scientists are trained from early in their careers and throughout about what constitutes misconduct. We take refresher courses and sign statements when we submit research papers regarding the validity of our work. Every researcher knows that being accused of misconduct can instantly ruin a career. In my years as an academic researcher, I have never known of anyone who was even accused of scientific misconduct. I'm not saying that it doesn't happen, but there is a big difference between a paper mill in China that is duplicating figures in different publications and an established researcher whose lab has consistently

published quality papers and is supported by NIH funding. It is improbable to think that Drs. Wang/Burns have systematically produced fraudulent research and manipulated data for research publications and grant applications without a colleague, collaborator, or reviewer detecting it for 15+ years.

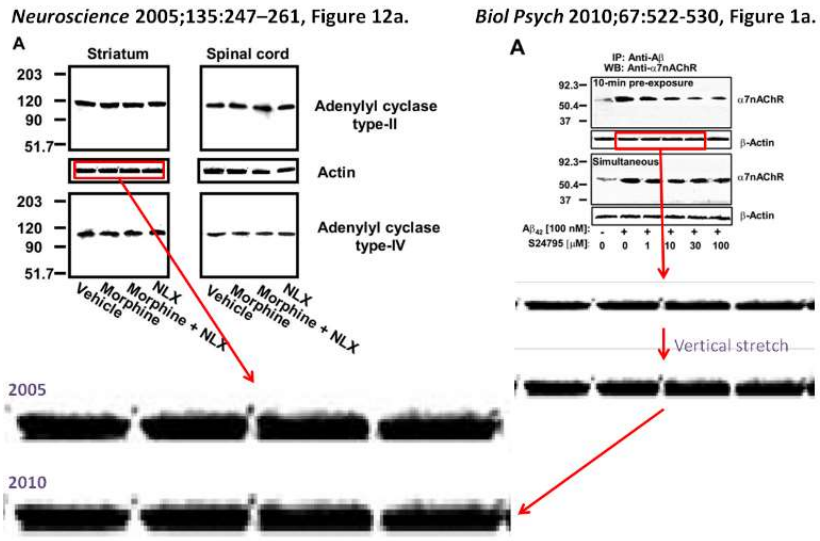
Response to specific critiques in the CP:

https://www.regulations.gov/document/FDA-2021-P-0967-0003

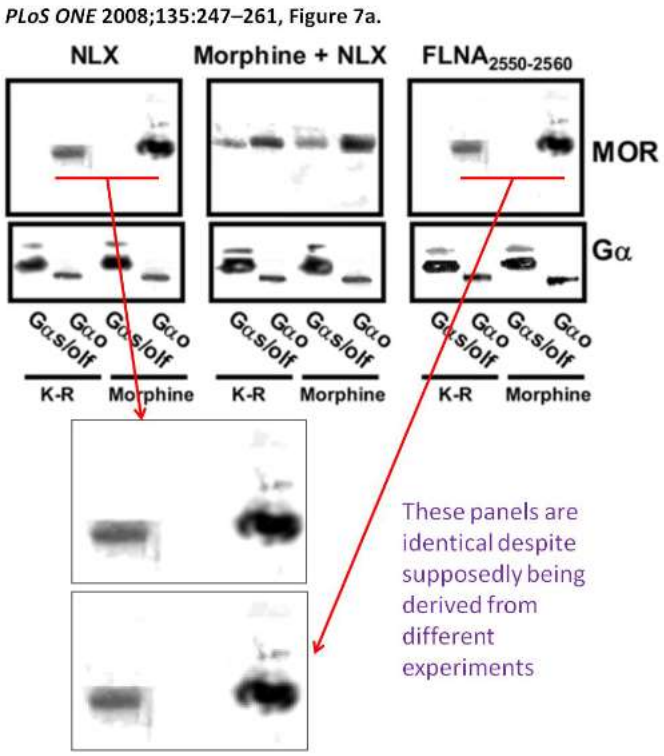
Example #1: “Manipulated WB”. The CP claims that different WBs were “spliced together” to make a figure in a 2005 paper. They point out a “bowtie” effect of several bands as supporting evidence. However, anyone with WB experience has seen this bowtie effect. In theory, it can be caused by proteins sticking to the sides of the wells or a small amount of extract entering the space between the wells and the plates that support the gel on each side. This effect is often seen when the top of the gel is exposed to air too long prior to loading.

*Neuroscience* 2005;135:247–261, Figure 5a.

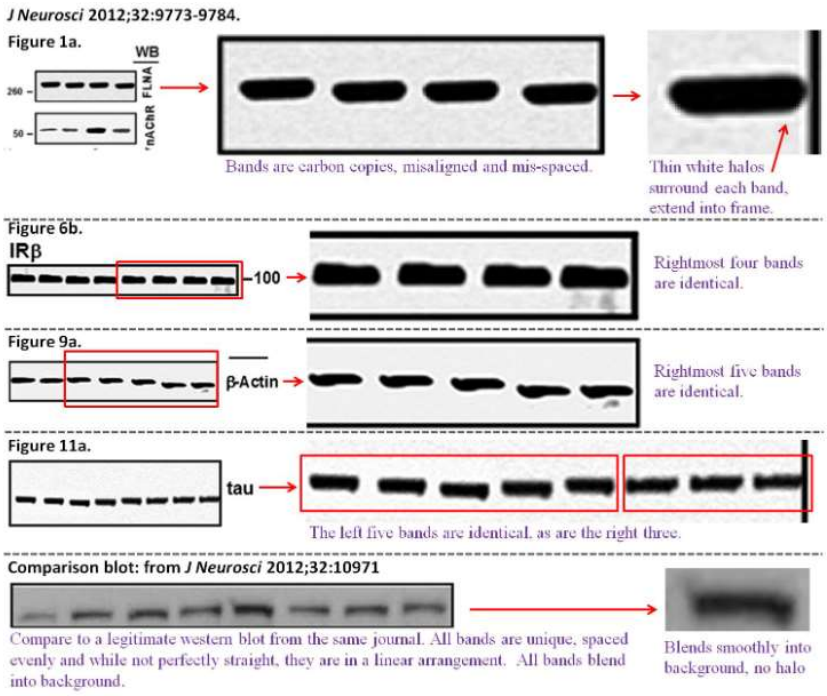

Example #2: “Falsified WB”. This claim states that the same beta-actin control WB was used in 2 different papers published 5 years apart. The CP takes a beta-actin WB, which is low resolution, stretches it vertically, then blows it up and compares it to a WB published 5 years earlier. Although they claim the blots are identical, to me they look completely different. For example: 1) the left side of the band in lane 1 of the 2005 publication has a notch that is not in the 2010 WB; 2) band images between lanes 2 and 3 are clearly different; 3) the right sides of the bands in lane 4 are also different. I also have difficulty taking this claim seriously. The labs likely ran many beta-actin WBs between the 5-year timeframe the 2 papers were published, but it is claimed they somehow elected to risk their scientific careers to include 4 control lanes from a previous publication? A new beta-actin WB could be run in half a day. This accusation suggests that the scientists who contributed to the CP are not very experienced in molecular biology.



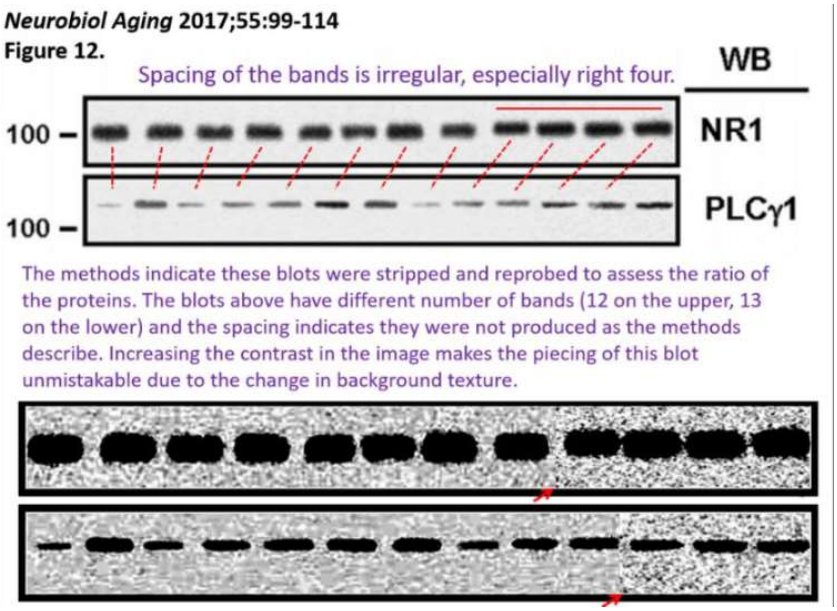

Example #3: “Reused WB”. This claim takes 2 WB panels of a figure and compares them side-by-side, suggesting they are identical. This claim could have some credibility, though it is more likely a mistake in constructing the figure for publication than scientific misconduct. Although I can’t say for sure that the 2 WBs in question are indeed the same, they do look very similar, though one is a slightly darker exposure. When WBs are exposed to film, we generally get several exposures from light to dark. If the films aren’t labelled properly, things can get mixed up when the graduate student/post-doc generates the figure for publication, especially if two of the WBs look similar. If the 2 blots are indeed the same, the PI, other authors, or reviewers probably should have asked if this was indeed an error. I’m inclined to think this was an error in making the figure and not intentional since there is nothing covert going on here- the 2 WBs are basically side-by-side in the same figure.




Example #4: “Band insertion into WBs”. The concerns raised here include: 1) irregular spacing between lanes; 2) FLNA bands not looking correct since it is a large (290 kDa) protein; 3) bands looking identical between lanes; and 4) white halos around bands. In my opinion, this claim is completely baseless and shows a lack of experience of the scientists that generated the CP. Irregular spacing between bands is routinely seen with self-poured gels. It is explained by the teeth of the comb used to cast the gel not being completely straight when the gel is polymerized. The claim that FLNA shouldn’t run very far in a gel because of its large size is inaccurate because proteins will run as far through a gel as a current is applied. Longer gels could have been used. Also, the researchers could have used gradient gels that allow for larger proteins to be better resolved in the gel. As far as the control beta-actin bands looking identical, they are basing this assumption on a common “tadpole-like” appearance of the bands. However, this tadpole effect is commonly seen if the gel is polymerized next to an air current (e.g. in a fume hood). The air current can cause the sides of the wells to lose moisture during polymerization resulting in a tadpole-like effect on the same sides of the wells of each lane, which is seen here. As far as halos around bands, most films used to expose WBs have a threshold level of exposure that is designed to limit background. You often see this halo effect directly on the exposed film, especially with stronger bands. The white halo could also be a compression effect, caused when the figure levels were adjusted for publication.

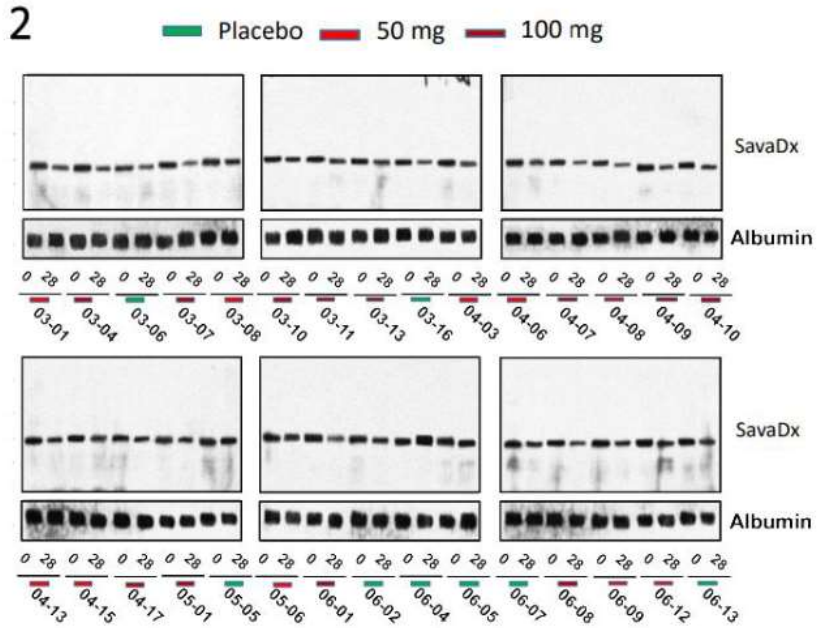



C3.2 “Evidence of data manipulation from human tissue”. It is claimed that Figure 12 of a 2017 paper shows a WB with 12 lanes for the control protein NR1 and 13 lanes for PCLgamma1. It is also suggested that WBs from different experiments were spliced together to make the figure. The 12/13 lane issue is an obvious error that likely occurred when making the figure for publication, but in no way rises to the level of scientific misconduct. As far as splicing together 2 different WBs, please refer to Response to General Concern #1 above about manipulating low-resolution figures and spliced WBs. I honestly don’t see much evidence of splicing for the top WB. Also, on the bottom WB, the change in pixels appears to wrap around the top of the band on the left side of the proposed splice site. It would be difficult to crop the figure this way. Another thing to point out is that the 3 lanes on the right of both WBs are running lower in the gel than the band directly to the left, which is slightly higher than the other bands to its left. However, the proposed splice sites are 4 lanes from the right on the top WB and 3 lanes from the right on the bottom WB. These factors support that the figure was not generated by splicing 2 WBs spliced together.



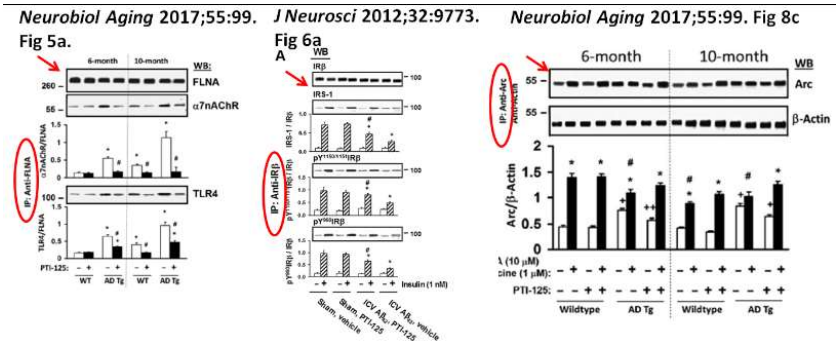

Appendix items/Additional areas of concern:

Claim #4: It is claimed that the top WBs cannot be FLNA based on the banding pattern. Please see Response to Example #4 above. Also, note that Albumin was used as the control protein here, which is a comparatively large (66 kDa) for a control protein and suitable for analysis of large proteins, such as FLNA.




Claim #6: It is claimed that several papers between 2010-2017 don't show control WBs for co-immunoprecipitation experiments that evaluate the interaction of beta-amyloid with alpha7-nicotinic acetylcholine receptors. This claim is true, but there is a very good reason for it. Beta-amyloid are small (10 kDa) peptides of 40-43 amino acids in length. Their small size makes them very difficult to analyze

by WB, other than running specialized gels that would likely preclude the analysis of larger interacting proteins, such as alpha7nAChR (60+ kDa). Most companies pre-validate their antibodies for use in certain applications, such as immunoprecipitation experiments.




E.2 Additional suspicious WBs (1) and probable band duplication (2):

1) Suspicious WBs. The CP shows several examples (pages 30-31) of control bands looking the same between WBs suggesting that the researchers must have duplicated these images. To me, this is a ridiculous claim. I see clear differences between the bands in question and I think most others do as well.

2A) Probable band duplication. Regarding the white halo around bands on WBs, see Response to Example #4 above.

2B) FLNA bands look identical (page 33). The CP highlights 3 FLNA bands that they claim are duplicated, but I see clear differences. For example, differences are seen with the bottom of the right and middle bands and top of the right bands.

2C) Five IRbeta bands look identical (page 34). There are clear differences in the sizes of the notch at the bottom left of each band, as well as the slopes of the bands on the top right.

2D) Clipping multiple blots together (page 35). The clip effect that is seen when the proteins run through an air bubble in the gel, which is common with self-poured gels.

2E) Tampering of WBs (pages 37-39). It is claimed that bands are inserted into various WBs in a 2006 Nature Medicine publication. This claim is borderline ridiculous and is likely why it appears at the end of the CP. Dr. Wang is first author on this publication, so the implication being made is that he started manipulating data early in his career and continues to do so today as head of an academic laboratory. Firstly, Nature Medicine is an outstanding journal, so the data was thoroughly vetted by experts in the field prior to publication. See Response to General Concern #1 regarding manipulation of low-resolution WB images and drawing conclusions. Also, several of the examples shown as evidence of data manipulation support the opposite. For example, on page 38 the background pixels around the bands are irregularly shaped. It is hard to believe that Dr. Wang would have cropped each of the bands for insertion as irregular shapes (which would be difficult in 2006) instead of just using a box shape.

Also, if differences in background pixels are evidence of data manipulation, why are there also irregular shaped pixels around the text in the figures? Were they copied from other figures as well? It is obvious that the differences in background pixels observed are the direct result of the scientists who constructed the CP altering the contrast and brightness of already low-resolution figures.

Specific Claim #2 made by the Citizen's Petition:
Filamin A (FLNA) has never been studied outside of Dr. Wang and never been linked to the brain.

Response:
Multiple peer reviewed journals have documented the link between FLNA and the brain. Examples are provided below:

Filamin A inhibition reduces seizure activity in a mouse model
https://www.science.org/doi/10.1126/scitranslmed.aay0289

Targeting Filamin A Reduces Macrophage Activity and Atherosclerosis

https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.119.039697

This inflammation is in turn linked to Alzheimer's disease:
Atherosclerosis and Alzheimer - diseases with a common cause? Inflammation, oxysterols, vasculature

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3994432/